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Award Number: W81XWH-08-1-0689

TITLE: Role of a Novel ER Coactivator in Control of Cell Prolifertion and Tamoxifen Resistance

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REPORT DATE: October 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

x Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
14-10-2009	Annual Summary	15 September 2008 – 14 September 2009

4. TITLE AND SUBTITLE
Role of a Novel ER Coactivator in Control of Cell Proliferation and Tamoxifen Resistance

5a. CONTRACT NUMBER
W81XWH-08-1-0689

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California Davis
Davis, CA 95518

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Estrogen receptor is a key figure in the process of breast cancer development. Being up-regulated in about two thirds of all breast cancers it is known to promote estrogen-dependent cancer cell proliferation and as a result tumor growth. It is still currently unclear what machinery is involved in transducing this activation signaling to target genes. The mediators that are able to integrate ER-dependent effects into the cell cycle machinery are the focus of the proposed project. A novel protein ATAAB discovered in our laboratory comprises a number of features that make it a possible target of our research. The goal of my training program is to find its place in a complicated web of protein interactions, gene networks and signaling that control cell proliferation. We were able to demonstrate that ATAAB (ANCCA) is highly overexpressed in breast tumors and its expression correlates with the progression of the disease. Our experiments with breast cancer cell lines showed that ATAAB (ANCCA) is essential for cell proliferation and survival.

15. SUBJECT TERMS – None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

ER alpha signaling is known to promote dramatic changes in gene expression profile and trigger cell proliferation, the detailed mechanisms and key mediators of this activation cascade still remain unknown. Identification of main players involved in transmitting the signal from ER to key cell cycle regulators such as cyclin D1 and E2Fs will provide better understanding of estrogen-dependent cancer cell proliferation and thus reveal new potential targets for therapy. Preliminary studies strongly suggest that recently identified novel protein ANCCA (ATAAB) may be one of such mediators of ER-dependent proliferation. It was demonstrated that expression of ANCCA (ATAAB) is tightly controlled by estrogen and proto-oncogene ACTR/AIB1, ATAAB can also directly associate with ER alpha, ACTR and activator E2Fs. Taken together all these data suggest an important role in estrogen-induced proliferation, thus ANCCA (ATAAB) may function as ER-coactivator to promote hormone-induced gene expression and cancer cell proliferation.

To reflect the nature of this novel protein and its tight association with cancer ATAAB was given a new name ANCCA, which stands for AAA+ Nuclear Coregulator Cancer Associated.

BODY

Task 1: To generate stable sub-lines of MCF7 cells that allow inducible down regulation of estrogen-dependent ANCCA expression.

To test whether E2-dependent activation of gene expression and proliferation occurs through ANCCA activation it was proposed to generate stable sub-lines of MCF7 cells that allow inducible down regulation of expression of this protein. One of the reasons to use a stable cell line was the difficulty of performing transient siRNA transfections with this cell line. After trying several different reagents we were able to optimize the conditions for the transfection procedure so that we could successfully knockdown ANCCA expression and obtain reproducible results. It was decided that the proposed experiments could be first tried using the new transient transfection system before proceeding with the generation of stable cell lines.

To test whether ANCCA is involved in the control of hormone-induced proliferation of this cell line we performed the following experiment. MCF7 cells were kept in hormone-deprived conditions for 3 days, treated with ANCCA or control RNAi. After the treatment with hormone every 2 days the cell number was counted and recorded. The results of this experiment presented in figure 1demonstrate that cells treated with ANCCA RNAi showed little or no proliferation as compared to the controls. Knockdown of ANCCA was able to abolish hormone-induced proliferation of MCF7 cells, demonstrating that ANCCA is involved in relaying proliferation signal from estrogen-bound receptor to downstream hormone-induced targets of the proliferation machinery of the cell.

Western blot analysis of MCF7 cells treated with ANCCA RNAi demonstrates that some of the genes critical for cell proliferation are affected by the knockdown of ANCCA. Some of the genes known to be involved in the control of cell proliferation and

also affected by ANCCA knockdown are cyc E1, cyc A2, cyc D1, E2F1. Some of these genes are shown in figure 1(b)

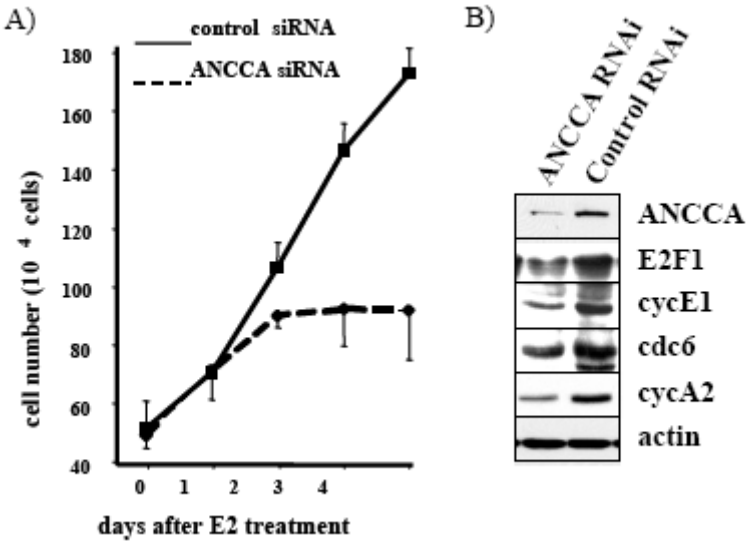

Fig 1. MCF7 cells were transfected with either control siRNA or ANCCA siRNA, maintained in hormone depleted medium, and 2 days later treated with E2 for cell proliferation (a); b) Western blot analysis demonstrating several key cell cycle regulatory genes affected by knockdown of ANCCA.

This way during the first funded period we were able to establish a reliable procedure for transfecting MCF7 cells with ANCCA siRNA with reproducible results. We were able to demonstrate that E2-dependent activation of gene expression and proliferation occurs through activation of ANCCA. This way our task one was about 30% completed. The next step will be to employ genome-wide analysis to identify more target genes affected by expression of ANCCA by using a microarray approach.

Task 2: To optimize the conditions for adenovirus infections of T47D breast carcinoma cell line and assess proliferation activity of the cells expressing ANCCA as compared to the cells infected with the control virus.

To optimize the conditions for adenovirus infection, T47D cells were grown under normal growth conditions and infected with various amounts of both ANCCA adenovirus and empty vector control. Cell toxicity and efficiency of infection were assessed by analyzing the morphology of the cells, followed by Western Blot analysis. The conditions were optimal if together with the high efficiency of infection expression of a set of proteins of control cells was not affected as compared to the mock infected cells.

Once the conditions were optimized, we studied the effect of ANCCA overexpression on the proliferation of T47D cells. Cells were plated in regular growth conditions, infected with adenovirus expressing ANCCA or empty vector for control. Every two days after infection the number of cells has been counted and recorded. Cells overexpressing ANCCA demonstrated increased rate of proliferation as compared to the control cells and also mock infected ones. The next step was to test whether ectopic

expression of ANCCA would result in hormone-independent proliferation of T47D cells. To address this question, T47D cells were plated in hormone deprived conditions that would normally inhibit their proliferation and the effect of ANCCA overexpression has been studied. Every two days after infection the number of cells has been counted and media changed for fresh. Cells ectopically expressing ANCCA demonstrated strong increase in cell proliferation as compared to both control and mock infected cells which showed slow or no significant proliferation (fig. 2).

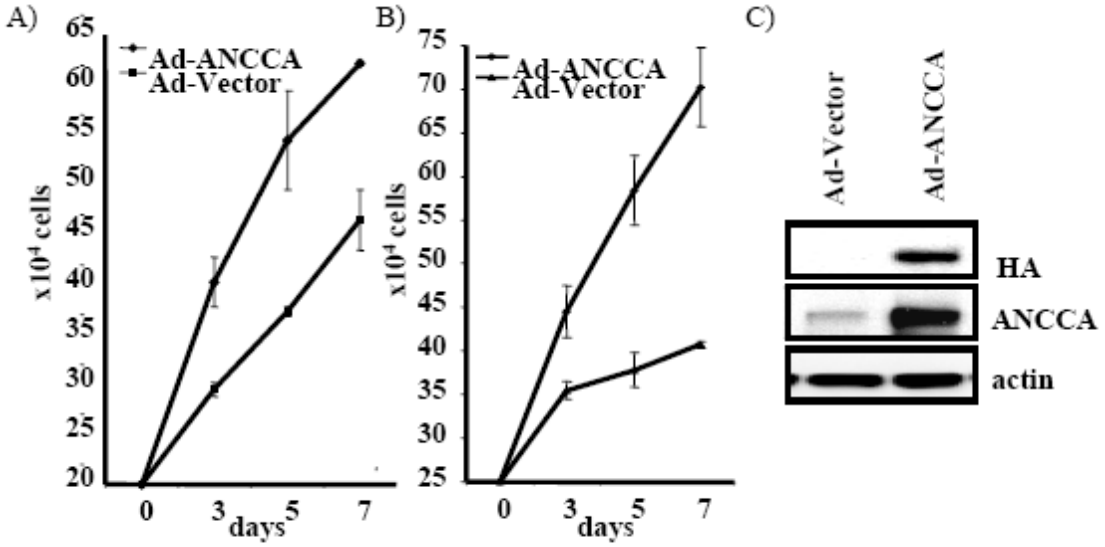

Fig 2. Ectopic expression of ANCCA not only promotes cell proliferation under normal growth conditions, but it is also allows T47D cells to proliferate in a hormone-independent way. A) Ectopic expression of ANCCA increases cell proliferation under normal growth conditions, B) Adenoviral mediated expression of ANCCA in T47D cells promotes hormone-independent growth, C) Adenoviral infection with Ad-ANCCA dramatically increases expression of ANCCA (Western Blot analysis using anti-HA and anti-ANCCA antibodies)

This way during the first funded period we were able to optimize the conditions for T47D adenovirus infection. We were able to demonstrate that ectopic expression of ANCCA not only increases rate of cell proliferation under normal cell growth conditions, but it also renders the cells insensitive to hormone-deprived conditions. T47D normally are very sensitive to the presence of hormone in the media to which they respond by increasing the rate of proliferation, while in media containing charcoal-dextrane stripped FBS (hormone deprived conditions) these cells become quiescent. Ectopic expression of ANCCA allows these cells to bypass the quiescence triggered by the absence of hormone in the growth media and continue to proliferate. This phenomenon indicates that ANCCA strongly promotes proliferation and that some of the anti-estrogen treatments might not be very effective for the cancer with high expression of ANCCA.

The next step will be to assess whether ectopic expression of ANCCA allows the cells to bypass the proliferation block caused by anti-estrogen treatment. Tamoxifen is the drug most commonly used for treatment of ER-positive breast cancer, the mechanism of action for this drug involves physical interaction with the receptor and thus preventing ER from activating transcription of its target genes. Our recent finding that overexpression of ANCCA allows the cells to proliferate under hormone deprived

conditions while the control cells show little or no significant proliferation allows to suggest that ectopic expression of ANCCA might also render these cells insensitive to the anti-estrogen treatment. This way task 2 is about 60% completed.

Task 3: To analyze a set of breast cancer samples for expression of ANCCA using IHC.

To analyze expression of ANCCA in breast cancer, a set of 200 human breast tumor samples was collected from UCD MC Tissue Repository. All of the specimens were collected within years 2000-2005 and preserved as paraffin blocs, patients were diagnosed with either DCIS or IDC. ER-positive and ER-negative cancer were equally represented in the collection of samples (about 100 samples of each type).

Before the analysis of the tumor samples, IHC procedure has been optimized for the ANCCA antibody. Different antigen retrieval buffers, incubation times and also different concentrations of the antibody have been tested. Once determined the conditions remained constant during the analysis of the entire set of tumors.

Immunohistochemical analysis of FFPE tissue samples was carried out for 120 samples. The tissue sample was considered positive for ANCCA expression if over 10% of epithelial cells on the slide showed positive nuclear staining. About 70% of the samples analyzed shows high expression of ANCCA, while normal and normal adjacent to cancer tissue generally show little or no positive staining for this protein. ANCCA is expressed in both ER-positive and ER-negative breast cancer, analysis of the remaining 80 samples will allow to conclude whether ANCCA expression is preferential to either of the two types of breast cancer (fig. 3).

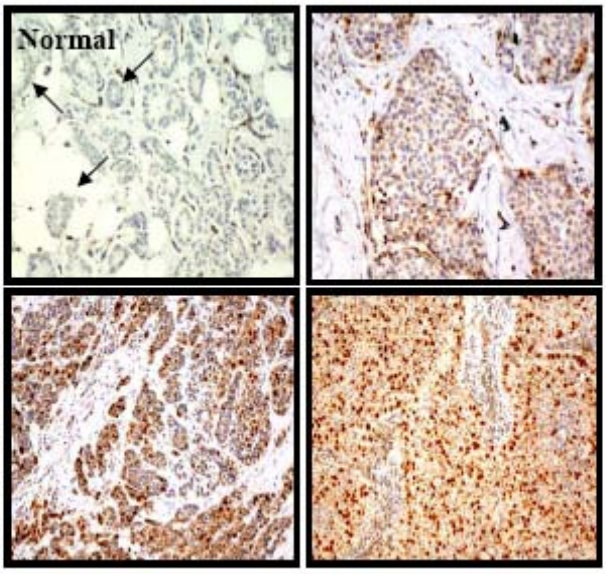

Fig 3. ANCCA is overexpressed in about 70% of all breast cancer samples analyzed (120 samples). Normal breast tissue shows little or no staining (normal ducts pointed by arrows); representative images of low, moderate and high expression of ANCCA are shown.

Statistical analysis for any potential correlation of ANCCA expression with certain stage in the development of cancer, ER status, proliferation marker Ki-67 or any other diagnostic markers still needs to be accomplished during the second and third funded period.

The number of breast cancer samples analyzed so far allows to conclude that ANCCA is greatly overexpressed in cancer samples as compared to the normal or normal adjacent to cancer tissue, we will continue our analysis to determine whether ANCCA expression could be used as a prognostic factor for this type of cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Conditions for siRNA mediated ANCCA knockdown for MCF7 cell line have been optimized, some of the genes significantly affected by the knockdown are…….
- Conditions for adenovirus mediated overexpression of ANCCA in T47D cells have been optimized. Ectopic expression of ANCCA led to increased cell proliferation in hormone-deprived conditions, while the cells infected with the control vector showed slow proliferation under these condition
- Collection of breast cancer tissue was made. 198 breast tumor samples from patients were obtained from UC Davis Tissue Repository
- The samples were analyzed for ANCCA expression using IHC analysis
- We were able to demonstrate that ANCCA is highly expressed in about 70% of all the samples analyzed while in normal tissue ANCCA expression is detected in small percentage of cells
- ANCCA expression correlates with expression of proliferation marker Ki-67 and disease progression. More aggressive high grade tumors show significantly higher levels of ANCCA expression then more differentiated low grade tumors
- ANCCA is highly expressed in both ER-positive and ER-negative breast cancer
- Depletion of ANCCA in breast cancer cell lines leads to a significant decrease in their anchorage-independent growth and increased levels of apoptosis, while ectopic expression of ANCCA promotes their proliferation

REPORTABLE OUTCOMES

Presentation at the UC Davis Medical Center Breast Cancer Mini-Symposium – August, 2009

Poster at the Fifteenth Annual Cancer Research Symposium – September, 2009

CONCLUSION

It was demonstrated that ANCCA is required for hormone-induced proliferation of MCM7 cells. Ectopic expression of ANCCA in T47D cells results in significantly increased rate of proliferation of this cell line and it also allows hormone-independent proliferation.

We were able to demonstrate that ANCCA is overexpressed in about 70% of breast tumors tested while normal tissue shows little or no expression of this protein. Further analysis needs to be done to understand if ANCCA expression is associated with ER-negative or ER-positive cancer.

ABBREVIATION

ANCCA (ATAAB) – AAA+ Nuclear Coregulator Cancer Associated
ER alpha - Estrogen Receptor alpha
IHC - Immunohistochemistry
FBS – Fetal Bovine Serum
FFPE – Formalin Fixed Paraffin Embeded
E2 – synthetic analog of estrogen